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EQUINOS PORTADORES de *Streptococcus equi* subespécie *equi*: PREVALÊNCIA, FATORES DE RISCO E CARACTERIZAÇÃO DE ALELOS *seM*

TESE DE DOUTORADO

Felipe Libardoni

Santa Maria, RS, Brasil
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EQUINOS PORTADORES de *Streptococcus equi* subespécie *equi*: PREVALÊNCIA, FATORES DE RISCO E CARACTERIZAÇÃO DE ALELOS *seM*

por

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Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Medicina Veterinária, Área de Concentração Medicina Veterinária Preventiva, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do grau de
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como requisito parcial para obtenção do grau de
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RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Medicina Veterinária
Universidade Federal de Santa Maria

EQUINOS PORTADORES de *Streptococcus equi* subespécie *equi*: PREVALÊNCIA, FATORES DE RISCO E CARACTERIZAÇÃO DE ALELOS *seM*

AUTOR: FELIPE LIBARDONI
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Santa Maria, 16 de janeiro de 2015.

A adenite equina é uma doença infecto-contagiosa que acomete o trato respiratório superior, sendo uma das principais doenças respiratórias de equinos. O agente etiológico dessa enfermidade é o *Streptococcus equi* subespécie *equi* (*S. equi*), responsável por aproximadamente 30% das notificações em todo o mundo. Os principais sinais clínicos da adenite são febre, secreção nasal e enfartamento de linfonodos, que ocorre pela dificuldade de fagocitose do *S. equi* por células de defesa devido a presença da cápsula de ácido hialurônico e proteína M. O entendimento sobre a epidemiologia, a análise de fatores de risco para adenite equina e o controle dessa enfermidade ainda são limitados. Estudos moleculares demonstram diferenças na extremidade 5' da sequência do gene (seM) codificador da proteína M de *S. equi*. Esta região do gene já foi utilizada na diferenciação de isolados por meio da caracterização de diferentes alelos. Por tudo isso, essa tese objetivou obter resultados de prevalência, e também análise de fatores de risco para adenite equina através de um desenho experimental para coleta de suabes nasais. Foram obtidos 1.010 suabes nasais de equinos sadios em 341 fazendas, de onde foram identificados 24 equinos positivos para *S. equi* em isolamento, que posteriormente foram confirmados por PCR e sequenciamento de DNA. A prevalência estimada por equino foi de 2.37%, e 20 fazendas foram consideradas positivas (5.86%). Na análise de fatores de risco, foi comprovado e quantificado estatisticamente que: número de eventos de aglomeração que os equinos participam (RR:1.06), o ato de compartilhar recipiente de alimento (RR:3.74) e ter tido diagnóstico positivo para adenite (RR:3.20) são fatores de risco relevantes para adenite equina. Estes resultados oferecem contribuições epidemiológicas importantes para a indústria de equinos e pode apoiar o controle da doença. Em paralelo, a região 5' terminal do gene seM das 24 amostras positivas foi amplificada por PCR e sequenciada para caracterização de alelos, sendo identificado o mesmo alelo (seM-61) em todas as amostras. Esses resultados evidenciam a hipótese de seleção natural de alelos aparentemente mais adaptados a sobreviver, persistir e se perpetuar na população estudada.

Palavras-chave: *S. equi,* adenite equina*,* garrotilho, proteína M, cavalo, alelo.

ABSTRACT

IN EQUINE CARRIERS OF *Streptococcus equi subsp. equi*: PREVALENCE, RISK FACTORS AND CHARACTERIZATION OF *seM* ALLELES

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Santa Maria, january 16th, 2015.

Strangles is considered the main respiratory disease in horses. The etiologic agent is the bacterium *Streptococcus equi* subsp. *equi* (*S. equi*), responsible for approximately 30% of horse diseases worldwide notifications. The clinical signs of strangles are fever, nasal secretion and lymph node enlargement. The last one occurs due the incomplete phagocytosis of *S. equi* by defense cells because bacterial hyaluronic acid capsule and M protein (SeM). The epidemiology, the risk factors and the strangles control are poorly understood. The 5' end of the *seM* gene sequence has been used for isolate differentiation by characterization of the alleles. Therefore, this thesis aimed to study the prevalence of *Streptococcus equi* subsp. *equi* (*S. equi*) in healthy horses, the alleles frequency and the risk factors involved on equine adenitis. One thousand and ten nasal swabs were obtained from healthy horses from 341 farms. Twenty four horses were positive for *S. equi*, confirmed by PCR and DNA sequencing. The prevalence of *S. equi* per equine was 2.37%, and 20 farms were positive (5.86%). Risk factor analysis showed by confirming and quantifying statistically that: the number of agglomeration events that horses participate (RR: 1.6), the situation with food container shared (RR: 3.74) and the positive diagnosis for adenitis (RR: 3.20) are significant risk factors for Strangles. These results provide important epidemiological contribution to the equine industry and can give support for the disease control. In addition, the 5 'end of the gene *seM* was amplified by PCR and sequenced and allele characterized. It was found the same allele (SEM-61) in all the samples. These results support the hypothesis of natural selection of alleles apparently more suited to survive, persist and perpetuate in the population studied.

Keywords: *S. equi,* strangles, M protein, horse, allele.

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1 - INTRODUÇÃO

A criação de equinos no Brasil tem grande relevância econômica e social, representando uma grande parcela no agronegócio brasileiro. O país possui o terceiro maior rebanho equino do mundo, com um plantel de 5,43 milhões de equinos, dos quais 522.578 mil estão no Rio Grande do Sul, ficando o Estado em terceiro lugar entre as unidades da federação (BRASIL, 2014; COSTA et al., 2013).

O setor equídeo forma hoje uma importante cadeia do agronegócio, com forte interação dos setores ligados ao esporte, lazer e turismo, sendo uma das cadeias produtivas que oferece mais oportunidades de trabalho para médicos veterinários, e que vem conquistando posição de destaque na economia nacional e internacional. Além disso, atividades ligadas à equideocultura, sejam elas de esporte ou lazer, movimentam R$ 7,5 bilhões anualmente na economia brasileira gerado milhares de empregos (ESALQ, 2011; Brasil, 2014).

Dentre as enfermidades que afetam equinos, o segundo grupo com maior prevalência é composto pelas doenças que acometem o trato respiratório, como a adenite equina, responsável por aproximadamente 30% das notificações de enfermidades em equinos em todo o mundo (CHANTER, 1997). A adenite equina, também conhecida como garrotilho, é uma doença infecto-contagiosa aguda caracterizada por inflamação mucopurulenta do trato respiratório superior dos equinos (SCHILD, 2001). O termo garrotilho deve-se ao fato de que cavalos afetados e não tratados parecem estar sendo estrangulados (garroteados), devido ao aumento dos linfonodos retrofaríngeos e submandibulares, que obstruem a faringe (ANZAI et al., 1999).

Esta enfermidade é causada pela bactéria β-hemolítica *Streptococcus equi* subesp. *equi* (*S. equi*) do grupo C de Lancefield. Também pertencem a esse grupo *Streptococcus equi* subesp. *zooepidemicus* e *Streptococcus dysgalactiae* subesp. *equisimilis*, microrganismos relacionados geneticamente, porém com potencial patogênico diferenciado e frequentemente isolados de amostras clínicas como contaminantes secundários (TIMONEY, 2004).

O uso de antimicrobianos é recomendado no tratamento da fase inicial da adenite equina. Essa terapia é eficiente somente nessa fase, caracterizada por febre, depressão e secreção nasal mucopurulenta, prevenindo a formação de abscessos (HARRINGTON et al., 2002).

Em surtos, recomenda-se o isolamento dos equinos doentes e a vacinação do rebanho para evitar a disseminação da doença. No Brasil estão comercialmente disponíveis vacinas contendo como antigeno bactérias inativadas (bacterinas). Porém, elas não asseguram um controle satisfatório, pois conferem apenas proteção a cerca de 50% dos vacinados. Vacinas experimentais contendo proteína M e proteína associada ao hialuronato (HAP) de *S. equi* estão sendo desenvolvidas a partir de tecnologias de proteína recombinante, mas não têm demonstrado proteção satisfatoria em equinos, possivelmente por variações dessas proteínas (MEEHAN et al., 1998; CHANTER et al., 1999; SHEORAN et al., 2002).

A identificação de uma variação na região N-terminal do gene da Proteína M vem sendo empregada para diagnóstico da adenite, e utilizada como ferramenta epidemiológica na caracterização de surtos da doença através da caracterização de diferentes alelos (KELLY et al., 2006).

Os principais objetivos dos estudos atuais sobre adenite equina visam um melhor entendimento sobre a base molecular e fatores de virulência do agente. Além disso, a produção de vacinas mais eficientes também é alvo de pesquisa. Técnicas avançadas de biologia molecular serão possivelmente o caminho para melhorar a compreensão sobre a adenite equina e, assim, minimizar limitações e resolver problemas ligados a patogenia e controle da doença.

Devido à ausência de estudos referentes a prevalência de *S. equi* em equinos saudáveis por meio de um delineamento de coleta de amostras sem vícios e representativos a população estudada, somado a associação desses resultados a fatores de risco para adenite, bem como da identificação de alelos da proteína M em equinos portadores, este estudo objetivou isolar *S. equi* de amostras de equinos saudáveis do Rio Grande do Sul - Brasil, inferir a prevalência na população, associar fatores de risco a adenite equina, e caracterizar alelos com base no sequenciamento da região 5' do gene da proteína M.

2 - CAPÍTULO 1 - Revisão de literatura

2.1 Adenite equina

A equideocultura constitui hoje uma importante cadeia do agronegócio, com forte interação entre os setores ligados ao lazer, cultura, turismo, esporte e segurança. O Brasil possui o terceiro maior rebanho equino do mundo, com um plantel de 8,4 milhões de equídeos (FAO, 2013). Com isso os setores ligados à equideocultura ascendem à posição de destaque na economia nacional, representando uma quota significativa de geração de serviços e empregos, movimentando cerca de R$ 7,5 bilhões anuais e oportunizando 3,5 milhões de empregos (ESALQ, 2011; MAPA, 2014). Entre os fatores que acarretam perdas à criação equina estão as doenças infecciosas, em que onde o segundo grupo com maior prevalência é composto pelas doenças que acometem o trato respiratório dos animais.

A adenite equina, também conhecida como Garrotilho, é uma doença infecto-contagiosa aguda caracterizada por inflamação mucopurulenta do trato respiratório superior dos equinos (SCHILD, 2001) e responsável por aproximadamente 30% das notificações em todo o mundo (CHANTER, 1997). Esta enfermidade leva ao aumento de volume dos linfonodos retrofaríngeos e submandibulares, que obstruem a faringe e acarretam significativos prejuízos no desempenho do animal (ANZAI et al., 1999).

2.1.1 Etiologia

Essa doença é causada por *Streptococcus equi* subesp. e*qui* (*S. equi*), uma bactéria Gram-positiva, encapsulada, β-hemolítica do grupo C de Lancefield, com morfologia de coco que forma longas cadeias de forma irregular. Em agar sangue, as colônias tem aspecto mucóide, cor de mel e apresentam uma ampla zona de hemólise. *S. equi* tem uma relação fenotípica e genética com *S. equi* subesp. *zooepidemicus* (*S. zooepidemicus*), ambos considerados espécies distintas até 1984 (FACKLAM, 2002), e também com *S. equi* subesp. *ruminatorum* (*S. ruminatorum*) (FERNANDES et al., 2004). Acredita-se que o *S. equi* possa ter evoluído do ancestral *S. zooepidemicus*, que é causador de doenças em equinos e também em outras espécies de animais, incluindo o homem (HOLDEN et al., 2009). Estes mesmos autores relatam que a complexa interação de especialização patogênica e o intercâmbio

genético entre *S. equi*, *S. zooepidemicus, S. ruminatorum* e *S. pyogenes* continua a influenciar a evolução desses estreptococos importantes.

As diferenças fenotípicas entre as subespécies de *S. equi* são pequenas e convencionalmente são detectadas por meio de testes bioquímicos, principalmente a fermentação de carboidratos. Utiliza-se lactose, sorbitol, trealose e fator CAMP como critério para a diferenciação dessas três subespécies. *S. equi* não fermenta nenhum destes carboidratos enquanto *S. zooepidemicus* fermenta lactose e sorbitol (KUWAMOTO et al., 2001), e *S. ruminatorum* apresenta reação CAMP positiva com *Staphylococcus aureus* (FERNANDES et al., 2004). Porém, muitas vezes a caracterização fenotípica tradicional não é capaz de diferenciar as subespécies devido à existência de cepas de *S. equi* atípicas fermentadoras de trealose, lactose ou ambos (GRANT et al., 1993). Com isso, métodos moleculares vêm ganhando espaço como ferramenta para diferenciação de subespécies, bem como diferenciação de isolados. Esses métodos envolvem técnicas de sequenciamento da região intergênica 16S-23S para identificação das subespécies de *Streptococcus* do grupo C de Lancefied (CHANTER et al., 1999), caracterização molecular da proteína M (WALKER & TIMONEY, 1998), técnicas de polimorfismo de DNA amplificado ao acaso (RADP-PCR) e eletroforese em gel de campo pulsado (PFGE) em investigação de surtos (GONZALEZ-REY et al., 2003), PCR multiplex para caracterização de espécies (ALBER et al., 2004) e sequenciamento do gene hsp60 (SILVA et al., 2007).

2.1.2 Proteína M de *S. equi*

Em uma revisão sobre as bases moleculares da infecção por *S. equi*, HARRINGTON et al. (2002) agruparam os fatores de virulência, conforme a função exercida, em categorias como a aderência bacteriana, evasão do sistema imune e aquisição de nutrientes, embora alguns dos fatores tenham múltipla função. Dentre esses fatores de virulência da bactéria, a proteína M, de 58 kDa codificada pelo gene SeM, tem especial importância (HARRINGTON et al., 2002). Esta foi caracterizada pela primeira vez por GALÁN & TIMONEY (1987), que clonaram seu gene e expressaram em *Escherichia coli*. A proteína M tem aspecto de fímbria que se projeta a partir da parede celular bacteriana, possui característica ácido resistente (GALÁN & TIMONEY, 1987), e atividade de aderência e antifagocítica, inibindo a deposição do componente C3b do complemento na superfície bacteriana. Essa também impede a ligação e inativa o fibrinogênio e a imunoglobulina G, inibindo a fagocitose por neutrófilos e macrófagos (BOSCHWITZ & TIMONEY, 1994; MEEHAN et al., 2000).

A proteína M vem sendo utilizada para diagnóstico da Adenite, além de ser uma candidata promissora a antígeno vacinal (TIMONEY & MUKHTAR, 1993), embora a utilização de proteína M purificada como antígeno vacinal não tenha conferido proteção significativa em equinos vacinados após serem desafiados com *S. equi* (SHEORAN et. al., 2002). Até então acreditava-se que a sequência do gene da proteína M de diferentes isolados de *S. equi* era altamente homogênea e conservada (GALAN & TIMONEY 1988), contudo dados recentes identificaram variações na região N-terminal dessa proteína (ANZAI et al., 2005). Em seguida KELLY et al. (2006) demonstraram o potencial para a exploração da variação dessa região, utilizando-a como ferramenta epidemiológica na caracterização de surtos da doença produzida por *S. equi*. Além disso, CHANTER et al. (2000) também relataram um truncamento (deleção de nucleotídeos que codificam a região N-terminal) da proteína M em equinos portadores.

O sequenciamento da região variável da proteína M, em surtos de adenite no Reino Unido nos anos de 2007 e 2008, permitiu identificar uma mudança nas frequências alélicas entre as cepas circulantes neste período, sugerindo pressão seletiva nesses isolados (IVENS et al., 2011). Além disso, PARKINSON et al. (2011) demonstram evidências de mutações no gene SeM que podem levar ao surgimento de novos alelos geograficamente relacionados. Estes afirmam que o sequenciamento do gene SeM é uma ferramenta útil para a elucidação da epidemiologia de adenite equina a nível regional e nacional. No Brasil, LIBARDONI et al. (2013) identificaram a presença de 15 diferentes alelos do gene da proteína M em isolados de *S. equi* de surtos de adenite equina no Estado do Rio Grande do Sul, e demonstraram que existem diferenças nas frequências alélicas em cepas isoladas de equinos da raça Puro Sangue de Corrida (maior freqüência do alelo sem-61) e da raça Crioula (maior freqüência do alelo sem-115).

Essas variações na região N-terminal alteram a conformação de epitopos e determinam mudanças na virulência, influenciando diretamente a resposta imunológica mediada por IgA e linfócitos T (TIMONEY et al., 2010). Isso facilita a colonização da mucosa por *S. equi* (TIMONEY et al., 2010). Além disso, *S. equi* produz endopeptidases (IdE e IdE2) que clivam IgG, reduzindo a opsonização que auxilia na fagocitose (LANNERGARD & GUSS, 2006; HULTING et al., 2009). Estudos demonstram que a inclusão de IdE e IdE2 aumentam a eficácia em vacinas de subunidade, demonstrando a importância dessas enzimas para evasão do sistema imune e o desenvolvimento de imunidade protetora contra *S. equi* em equinos (GUSS et al., 2009).

2.1.3 Epidemiologia

A infecção por *S. equi* ocorre através da inalação e/ou ingestão do micro-organismo, seguido pela fixação deste no epitélio nasofaríngeo e migração para os linfonodos regionais (TIMONEY, 2004). Os principais sinais clínicos da adenite são febre, corrimento nasal e enfartamento de linfonodos, que resulta da dificuldade de fagocitose por células de defesa devido a presença da cápsula de ácido hialurônico e proteína M (BOSCHWITZ & TIMONEY, 1994; ANZAI et al., 1999).

Em surtos de adenite, são de grande importância os fatores predisponentes na transmissão e disseminação da doença, tais como fatores estressantes como desmame, viagens, alterações climáticas bruscas, doenças concomitantes, superlotação, deficiências nutricionais, parasitismo, transporte, idade e estação de monta onde são agrupados animais de diferentes origens.

Os equinos de sobreano são mais predispostos a desenvolver adenite, seguidos de potros desmamados e adultos (YELLE, 1987). Outro importante fator na disseminação do agente etiológico da adenite é a existencia de animais portadores assintomáticos, que geralmente abrigam a bactéria nas bolsas guturais. O diagnóstico nesses casos é complexo, pois a forma mais eficiente é o exame endoscópico das bolsas guturais, que é utilizado para coleta de material a ser enviado para diagnóstico laboratorial por PCR e cultura (NEWTON et al., 2000), e vizualização de condróides (concreções esféricas), formados quando ocorre a drenagem de pus dos linfonodos abscedados para o interior das bolsas guturais (NEWTON et al., 1997).

O inquérito de prevalência de *S. equi* em equinos saudáveis com um delineamento amostral, e a associação da prevalência aos fatores de risco para adenite equina ainda não foram relatados até o momento. Estudos que demonstrem a prevalência de *S. equi* em equinos portadores aparentemente saudáveis com associação a fatores de risco ao desenvolvimento de adenite equina são escassos. Embora tenham sido demonstrados índices de positividade em estabelecimentos de criação de equinos no Canadá (18,4% em isolamento de equinos com sinais clínicos) por CLARK, et al., (2008), na Arábia Saudita (28% de equinos sorologicamente positivos) por AL-GHAMDI, (2012), e no Brasil sem inferir prevalência por LIBARDONI et al., (2013), esses relatos não tiveram um delineamento amostral que pudesse inferir um índice epidemiológico de prevalência, uma vez que utilizaram amostras por conveniência.

2.1.4 Tratamento e controle

O uso de antimicrobianos no tratamento da adenite equina é controverso. Embora *S. equi* seja sensível à maioria dos antimicrobianos *in vitro* (KIRINUS et al., 2010), essa terapia é eficiente somente na fase inicial da doença, caracterizada por febre e depressão, previnindo a formação de abscessos (HARRINGTON et al., 2002). No entanto, o tratamento nessa fase pode inibir o desenvolvimento de imunidade (SWEENEY et al., 2005). Por outro lado, nos casos em que ocorre o enfartamento de linfonodos, o tratamento geralmente é ineficiente devido a vascularização insuficiente no abscesso para permitir a penetração de antibióticos em níveis adequados (HARRINGTON et al., 2002). A recomendação nesse momento é acelerar o processo inflamatório (maturação do abscesso) com a aplicação de pomadas rubefascientes para antecipar a supuração dos abscessos (SWEENEY et al., 2005).

Nos casos de surtos de adenite, o manejo dos equinos doentes é o fator mais importante no controle da disseminação da doença. Animais doentes devem ser imediatamente separados e submetidos ao tratamento de acordo com a evolução do caso, e os equipamentos e instalações devem ser devidamente higienizados e desinfectados. (SWEENEY et al., 2005).

A imunização com vacinas contendo bactérias inativadas (bacterinas) como forma de prevenção da enfermidade não asseguram um controle satisfatório, pois conferem apenas cerca de 50% de imunidade nos animais vacinados (TIMONEY & EGGERS, 1985). Por razão desta baixa eficiência, foi desenvolvida uma vacina de inoculação intranasal produzida com uma cepa de *S. equi* atenuada denominada *Pinnacle* (WALKER & TIMONEY, 2002). Esta atenuação foi realizada por mutações induzidas que tornaram esta cepa incapaz de produzir cápsula. Porém, a reversão dessas mutações e o retorno da produção de cápsula pela bactéria é possível, revertendo a virulência e desenvolvendo doença nos animais (WALKER & TIMONEY, 2002). Com isso a utilização dessa vacina torna-se arriscada devido a reversão da virulência.

Visando entender os baixos índices de proteção conferidos por vacinas utilizadas no Rio Grande do Sul, MORAES (2009) estimou o Índice de Reatividade Cruzada (IRC) de 10 cepas de *S. equi,* isoladas de 35 equinos com garrotilho da região sul do Estado, entre elas e com duas vacinas comerciais, comprovando que as cepas apresentaram IRCs indicativos de relativa homogeneidade antigênica entre a maioria delas, mas que os baixos IRCs, com duas vacinas, poderiam explicar a baixa proteção conferida pelas vacinas em condições de campo.

Vacinas experimentais estão sendo desenvolvidas a partir de tecnologias de DNA recombinante. Imunógenos contendo a proteína M de *S. equi* como antígeno têm demonstrado eficiência em estudos com ratos desafiados pós vacinação, mas ainda não demonstram proteção satisfatória em equinos (MEEHAN et al., 1998; SHEORAN et al., 2002). Da mesma forma, a vacinação com proteína associada ao hialuronato (HAP), que forma a cápsula do *S. equi*, demonstrou proteção em camundongos, mas não conseguiu impedir o desenvolvimento de adenite em equinos vacinados (CHANTER et al., 1999). Além disso, também foram testados extratos proteicos de *S. equi* encapsulados em nanopartículas testados como antígeno vacinal por via intranasal e conferiram proteção em camundongos (RODRIGUEZ et al., 2012; FIGUEIREDO et al., 2012).

3 - CAPÍTULO 2

Prevalence of *Streptococcus equi* subsp. *equi* in horses and associated risk factors in the State of Rio Grande do Sul, Brazil

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ABSTRACT

The aim of this study was to estimate the prevalence of equine adenitis and identify the risk factors for the disease through a cross-sectional study of nasal swabs. Nasal swabs (n=1,010) of healthy equines from 341 farms were plated on 5% blood agar; of these horses, 24 were identified as positive for *S. equi* through isolation, PCR and DNA sequencing. The estimated prevalence for individual animals was 2.3% and for herds, 5.86%. The statistical analysis identified the following as risk factors: number of agglomeration events that were attended by the equines (PR: 1.06), sharing of food containers (PR: 3.74) and having a positive diagnosis for adenitis (PR: 3.20). In addition to this, there was also evidence of a higher rate of positivity in quarter horses. These results constitute an important epidemiological contribution to the horse industry and may support the future control of the disease.

Keywords: epidemiology, strangles, equine, respiratory, adenitis

1. Introduction

Streptococcus equi subsp. e*qui* (*S. equi*) is a microaerophilic bacterium, Gram-positive coccus, encapsulated and in β-hemolytic group C of Lancefield (Quinn et al., 2011). This pathogen is the etiologic agent of equine adenitis, also known as strangles, an acute infectious disease of worldwide distribution, characterized by purulent inflammation of the upper respiratory tract of horses (Schild, 2001). This disorder presents with an increasing volume of retropharyngeal and submandibular lymph nodes that obstruct the pharynx and cause significant harm to the animal's performance and also causes empyema of the guttural pouches (Anzai et al., 1999).

S. equi infection occurs through inhalation and/or ingestion of the microorganism, followed by the microorganism fixing in the nasopharyngeal epithelium and migrating to the regional lymph nodes (Timoney, 2004). The main clinical signs of adenitis are fever, runny nose and clogging of the lymph nodes (Boschwitz and Timoney, 1994; Anzai et al., 1999).

Outbreaks of adenitis occur due to predisposing factors such as the stress of weaning, abrupt climate change, concomitant diseases, overcrowding, nutritional deficiencies, parasites, animal handling (agglomeration) and the breeding season, a period when animals of different origins are grouped (Moraes et al, 2009; Oikawa et al, 1995). Yearling horses are most prone to develop adenitis, followed by weaned foals and adults (Yelle, 1987).

Studies on the prevalence, incidence of *S. equi* and risk factors for the development of equine adenitis are very rare in the literature. So far, Clark et al. (2008) reported positivity by isolation (18.4%) in samples collected from guttural pouches, and AL-Ghamdi (2012) reported 28% of horses as seropositive. Thus, this study aimed to obtain results regarding the prevalence at the level of the animal and the herd, and also to conduct an analysis of the risk factors for equine adenitis through a cross-sectional study based on nasal swab samples.

2. Material and Methods

2.1 Isolation, PCR and sequencing of *S. equi*

A total of 1,013 nasal swab samples collected from healthy horses with the aim of isolating and identifying *S. equi* were seeded and cultured on agar with 5% sheep blood according to MacFaddin (2000) and Kuwamoto et al. (2001). To confirm a single agent, polymerase chain reaction (PCR) was conducted using the sense primer ASW73 (5'-CAG AAG AAA ACT TGC CGG TG-3 ') and antisense ASW74 (5'-ATT CGG TAA GAG CTT GAC CG-3 ') according to Kelly et al. (2006) after the extraction of DNA according to the CTAB (cetyltrimethyl ammonium bromide) protocol, which was preceded by a 5μL digestion with proteinase K (20 mg / ml) for 60 minutes at 37 °C by Sambrook and Russell (2001).

After the PCR reaction confirmed the gender and species of *S. equi*, the samples were sent in triplicate to ACTGene Molecular Analysis LTDA (Biotechnology Center, UFRGS, Porto Alegre, RS) for sequencing on an automatic ABI-PRISM 3100 sequencer genetic analyzer (Applied Biosystems), which was equipped with 50 cm capillaries and POP6 polymer, for confirmation of *S. equi*.

2.2 Study area and target population

Located in Brazil's southern region, the State of Rio Grande do Sul has an area of 268,782 km² (3.16% of the country) and is bordered by two countries: Argentina and Uruguay (Fig. 1). The state is divided into seven regions, which are subdivisions of Brazilian states that serve to group together various counties due to proximity and common agroecological characteristics (Fig. 1).

According to the official data from the Secretary of Agriculture, Livestock and Agribusiness of the State of Rio Grande do Sul (SEAPA-RS), there were ~103,180 equine farms registered on the database in 2013. Brazil has the fourth largest herd of horses in the

world, with more than five million animals (Parker, 2012). In 2013, the State of Rio Grande do Sul (RS), according to Costa et al. (2013), held approximately 522,578 animals, the second largest population of horses in the country.

2.3 Survey design and sample collection

A cross-sectional survey was performed to estimate the prevalence of *S. equi* in equines in the State of Rio Grande do Sul, as well as to identify risk factors. The sample procedure was performed in two stages. In the first stage, a predetermined number of properties that met the criteria of the target population of the study (the presence of at least one animal over six months of age) were randomly drawn. In the second stage, a pre-established number of horses over six months old were selected systematically.

The sample size and the calculation of sample size properties were determined according to Thrusfield (2007) and were performed as in Ausvet (Sergeant, 2013). The sample parameters were defined based on the number of properties that had at least one animal (N=103,180) enrolled in the Agriculture Defense System (SDA). For the calculation of the sample, the farms were considered the sample unit, and an a priori prevalence of 30% was assumed, with a confidence level of 95% and an absolute accuracy of 5%. The primary unit was randomly sampled. The farms with no equines when visited were replaced by the nearest property.

Based on the above parameters, the minimum number of primary units required was 323 farms. In order to have a safety margin, an additional 5% of farms were sampled, bringing this number to 341 (Table 1). After the number of primary units to be collected was determined, the number of secondary sample units (equines) was determined. To determine the minimum number of animals that needed to be collected per farm, an a priori herd prevalence (intra-herd) of 25% was assumed, and a simulation was conducted using the

Herdacc software program (Martins, 2004). The sample size chosen allowed for herd sensitivity and specificity of 100% (Almeida et al, 2006; Coggins et al, 1972; Issel and Coggins, 1979). Thus, in farms with fewer than 10 animals, all animals were collected; for those with more than 10 animals, 10 were sampled with a systematic selection procedure.

2.4 Questionnaire and interview

A questionnaire designed to gather information about the potential risk factors associated with the occurrence of *S. equi* infection at the studied farms is summarized in Table 2. The questionnaires were administered in April 2013. Particular regional vocabulary was considered in the structure of the questions. The structured questionnaire had 21 "close-ended" questions grouped into three main management categories: general farm characteristics, biosecurity, reproductive management, and farm sanitary conditions. It was previously tested with nonparticipating farmers to identify potential sources for misinterpretation and to further refine the questions. Each personal interview lasted 40–120 min; the farm owners/managers were interviewed face-to-face to complete the questionnaires. A copy of the questionnaire is available from the corresponding author upon request. In addition to the individual information on each animal, information was also collected on the species (equine, asinine or mule), breed, sex, age and pelage.

2.5 Data management

Epi Info 7 (CDC) was used to enter the data and to track data quality. Two separate databases were created, one for the questionnaire and the other for the animal information. For every one hundred questionnaires and laboratory results entered in the databases, 5% were randomly sampled and double-checked for data quality verification. The spatial location (GIS) of each sampled farm in the survey was determined with a handheld global positioning

system (GPS) and then plotted on a map using GIS software ArcView 10 (ESRI, Redlands, CA, USA).

2.6 Statistical analysis

The prevalence for *S. equi* was calculated at the animal and farm level. A 95% confidence interval was calculated using exact binomial confidence intervals (Clopper & Pearson, 1934). Furthermore, a correction for finite populations was applied (Isserlis, 1918).

All variables collected by the questionnaire and from the individual animal were tested for sufficient variability among biological plausibility of the outcome basis. The distribution of continuous variables was tested by histogram, mean, standard deviation and range. For categorical variables, analysis was performed based on the frequency distribution and bar charts. All statistical analysis was carried out with R language, v.3.1.1 (R Development Core Team, 2012). Variables were first screened based on the response rates and frequencies of the responses. Variables with large amounts of missing data ($> 10\%$) and limited variability ($<20\%$) were not included in the univariable analysis (n=6).

2.7 Univariable analysis

A logistic regression was used in this study. Two models were built: a farm model, where the outcome variable was the presence or absence of *S. equi* on the swab collected and where a farm that had at least one animal positive for *S. equi* is considered as positive, and an animal model, where the outcome variable was the same as in the farm model, but the unit of interest was each animal. For the farm model, a standard univariable analysis was first conducted using all 15 preselected variables; subsequently, all variables with a *p*-value ≤ 0.20 (Wald-type-III) were selected for inclusion in the multivariable analysis. For the animal model, all five variables were analyzed in multivariable fashion. Variance inflation factors

(VIF) were estimated to verify the relationships among all selected independent variables to check for potential collinearity, in which a coefficient > 2.50 was considered to be high. For significant associations, only the variables with a *p*-value less than 0.05 were considered for the multivariable model.

2.8 Multivariable analysis

For both models, the multivariable logistic regression model was built by manual forward selection with final manual backward elimination. During each round of model building, the best model chosen was the one with the lowest Akaike Information Criterion (AIC). The final model was adjusted using backward elimination to remove variables that had subsequently become nonsignificant ($p \geq 0.05$) during the forward selection process. Therefore, all variables in the final model were significant by the Type III Score statistic (when considering variables with $p \leq 0.05$ as significant). Confounding variables were checked and identified as present if regression parameters changed >0.25%. The goodness-of-fit of the final models was tested using the Hosmer-Lemeshow method (Dohoo et al., 2009), and the discrimination power was tested by means of the ROC curve.

3. Results

3.1 *S. equi* prevalence

The number of sampled nasal swabs examined for the presence of *S. equi* was 1.010 from the 341 farms, with 91,47% of the farms (each with 1 to 260 horses) having 1 to 10 positive animals among those sampled. A total of 24 equines were positive, the estimated animal prevalence of *S. equi* was 2.37% (CI 95%: 1.43%-3.31%), and at the herd level, twenty farms were considered positive, 5.86% (CI 95%: 3.37%-8.35%) (Table 1). There was

a difference in the proportion of positive farms and animals when the regions were considered (Table 1)

3.2 Risk factor analysis

For the farm version of the univariate logistic regression model, nine variables showed $p \leq 0.20$ (Table 3). The variance inflation factor showed no relevant relationship among independent variables. In the final multivariable model, three factors remained significantly associated with the presence of *S. equi* within the farms: "Number of agglomeration events to which the equine was sent," "Shared food container" and "Had strangles diagnosed on the farm" (Table 4). The risk of *S. equi* increased with the number of agglomeration events to which the farmer sends animals (PR: 1.06, $CI_{95\%}$: 1.04-1.10), and the farms whose horses shared food containers showed an increase in risk to approximated 3.7 in comparison to farms that did not allow shared food containers (PR: 3.74, $CI_{95\%}$: 1.61-8.70). There was also association with farms that had cases of strangles in the past year; these farms were 3.2 times more likely to have positive swabs than the farms that had not experienced this disease in the past year (PR:3.20, $CI_{95\%}$: 1.43-7.13).

For the animal model of the final multivariable analysis, only one factor remained significantly associated with the presence of *S. equi* in animals: "Equine breed" indicated that the quarter horse was the only equine race more likely to have *S. equi* isolated (PR: 7.58, $CI_{95\%}$: 2.78-20.64) (Table 5).

4. Discussion

To the best of the authors' knowledge, the prevalence of *S. equi* such as one based on a probabilistic sample and with identification of risk factors for equine adenitis has not yet been reported. Although positivity rates have been demonstrated in equine establishments in Canada (Clark, et al., 2008), Saudi Arabia (Al-Ghamdi, 2012), and Brazil (Libardoni et al.,

2013), these accounts have not had a sampling design that would allow for drawing inferences about epidemiological rates. In such cases, samples of convenience were analyzed, because they were from animals clinically suspected of infection with *S. equi*.

This study showed a prevalence of 2.37% of positive horses, and when analyzing at the herd-level, 5.86% of the herds were positive. Using isolates from the guttural pouch, Clark et al. (2008) found positive isolates in 18.4% of horses sampled in Canada between the years 1998 and 2003. Additionally, AL-Ghamdi (2012), in a serological evaluation, found 28% positivity; however, this study used samples from 181 asymptomatic animals from among 1343 horses belonging to 103 farms, and Mir et al. (2013) collected nasal swabs from 88 apparently healthy equines from five regions of India without observing *S. equi*. However, a prevalence of 21.59% for *S. zooepidemicus* was verified. In a study by the United State Department of Agriculture (Animal and Plant Health Inspection Service), nasal swabs collected from 5,976 healthy horses verified a prevalence of 0.05% for *S. equi* (Aphis, 2001).

Importantly, the difference between those results and the results of the present study mainly reflect the methods used to obtain the samples, because our samples were randomly collected from healthy horses. In a study by Clark et al. (2008), the samples were from diseased animals, which may explain the high incidence of positive animals. In the study by AL-Ghamdi (2012), samples from asymptomatic animals were also used, but serologically tested, indicating contact with *S. equi* and not necessarily that these equines are carriers. In addition, the presence of *S. equi* in all regions of the State of Rio Grande do Sul (RS) demonstrates the uniform geographic distribution of equine carriers of *S. equi*. The difference in prevalence among regions was tested by a chi-squared test and no significant differences were found (P=0.24) (Fig. 1).

It is important to note that Brazil has the fourth largest herd of horses in the world, with approximately 6 million animals (FAO, 2014). RS has the third largest horse herd in the

country with 535.682 horses, where the main Brazilian breeders of horses are located. In the analysis showed Fig. 1, the greatest number of positive samples is found in the region of greatest importance, due to it having the highest concentration of equines in the state. These results corroborate those found by Libardoni et al. (2013), in which a large number of strains of *S. equi* were isolated from this region. It is noteworthy that, although the farms in this region are highly technical, there is a large movement of animals among properties, and as demonstrated, this is a risk factor for the spread of disease and for disease control programs.

It was also observed that the risk of infection by *S. equi* in horses participating in crowd events with other animals increases to 1.06; that is, every event increases the risk of acquiring *S. equi* by 6%. According to Sellon (2013), the participation of animals in crowd events is an important factor in the transmission of *S. equi*.

In studies evaluating the frequency of M protein alleles of *S. equi*, the hypothesis has been raised that the frequency of some alleles in certain breeds of horses indicates better transmission or dissemination, because horses from different backgrounds participate in contests exclusive to the race and therefore have a common environment of movement and interaction (Parkinson et al, 2011; Libardoni et al, 2013.). Based on these results, the importance of preventive methods, such as vaccination of susceptible animals is stressed as a way to control the transmission of the agent.

In this study, a risk of 3.74 for *S. equi* infection was verified in horses that share the same feeder, while a risk of 3.20 was found for animals from properties that had previously had a diagnosis of adenitis. These factors may favor the onset of an outbreak because they are very important in the transmission (Prescott and Wright, 2000). Therefore, a risk of 2.58 was showed in proprieties that new animals are introduced, what could be attributed to equine patients or in incubation period of adenitis (Newton et al., 1997). A major source of infection on farms is the presence of asymptomatic animals (Sellon, 2013), because they share objects

with susceptible animals, especially those not previously exposed or not immunized (Yelle, 1987). Approximately 20% of the affected animals become asymptomatic, and that chance increases to 50% when also the empyema of the guttural pouch occurs (Newton et al., 1997).

In the analysis of the breeds of horses sampled (Table 5), a higher frequency of *S. equi* was observed in Quarter Horses. In Rio Grande do Sul, according to the survey, the breeders of this breed are less technical; that is, they have less monitoring of the animals by veterinarians. Furthermore, Quarter Horses participate more often in crowding events with animals because they are used mainly for equestrian sports characteristic of southern Brazil.

5. Conclusions

This investigation revealed isolates of *S. equi* on farms lacking current incidents of strangles disease. At the farm level, the presence of the infection was related to the number of events to which the horse owner had taken the animals, the sharing of food containers and a previous incidence of strangles. However, at the animal level, Quarter Horses may be more susceptible to cases of strangles. This paper provides information for a better discussion on the prevalence of *S. equi* based on a probabilistic sample, which has been noticeably lacking in the current disease literature. Finally, the results are an important epidemiological contribution to the horse industry and may contribute to future disease control.

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Table 1. Distribution of farms sampled by region and the number of animals sampled

Region	Total herds (N)	Total animals (N)	Positive herds (n)	Positive animals (n)
1.Northwest	17.829 (17%)	69728 (13%	8.47% (5/59)	5.12% (6/117)
2.Northeast	9.443 (9%)	58572 (11%)	6.45% (2/31)	2.43% (2/82)
3.Southwest	17.460 (17%)	149274 (29%)	3.44% (2/58)	0.7% (2/271)
4.West Central	11.235 (11%)	42401 (8%)	5.40% (2/37)	2.15% (3/139)
5.East Central	6.903 (7%)	27630 (5%)	4.34% (1/23)	1.03% (1/97)
6.Metropolitan	15.129 (15%)	82389 (16%)	4.00% (2/50)	3.00% (3/100)
7.Southeast	25.081 (245)	92584 (18%)	7.22% (6/83)	3.43% (7/204)
Total	103.080	522578	5.86% (20/341)	2.37% (24/1010)

Table 2. Questionnaire designed to gather information about potential risk factors associated with the occurrence of *S. equi* infection on the studied farms.

Subject	Factor/variables
General farm characteristic	Farm area extent and area designated as horse pasture. Main type of farm activity (hobby/reproduction/work/meat). Number of persons involved directly with horse labor. How much does the horse activity represent the reason for the family to stay on the farm? Does the farm have veterinary service?
Biosecurity	In the past year, has the animal been taken to an agglomeration event; to how many events; and was it at the regional level, state level, national or international level? Has any horse been brought to the farm in the last year, and if so, was it acquired at an event, from a dealer or from another farm? Does the farm have common-use utensils and infrastructure such as water and food containers, brushes, and dental rasps?
Reproductive management	What is the reproductive system used: artificial insemination or natural mating? If natural mating is used, is the stallion is raised on the property, bought, borrowed or rented?
Farm sanitary conditions	Are animals vaccinated against infectious disease? If injections are given, are disposable needles used, or not? When the veterinary service is on the farm does it clean and/or replaces gloves? Is any agglomeration event held within a maximum distance of 40 km from the farm?

Table 3. Definition and distribution of explanatory variables (farm model) retained in the univariable logistic regression analysis, using $P \leq 0.20$ as significant

Variables	No. of equine	Frequency (%) or median (S.D.)	P – value	PR (IC 95%)
Has strangles been diagnosed at the farm	340		0.003	4.17 (2.13-8.17)
Yes		23		
No		77		
Total farm area (ha)	340	77	0.13	1.00 (1.00-1.01)
Continuous				
Total number of equine	340	2	0.13	1.00(0.99-1.01)
Continuous				
Total area used to rise equines	340	45	0.04	1.01 (1.00-1.02)
Continuous				
Number of agglomeration events to which equines were sent	340	2	<0.001	1.05 (1.03-1.07)
Continuous				
Event at the regional level	340		0.04	
Yes		19		2.27 (1.16-4.45)
No		81		-
Have animals been introduced in the last year	340		0.02	
		259		2.58 (1.29-5.16)
Yes		42		-
No				
Shared food containers	340		<0.001	
Yes		34		3.17 (1.59-6.34)
No		65		-
Shared riding equipment	326		0.08	4.16 (1.03-4.55)
Yes		51		-
No		49		

Table 4. Multivariable logistic regression analysis of variables from the farm model associated with the isolation of *S. equi*.

Variables	Estimate (β)	*P* – value	PR (CI: 95%)
Intercept	2.80	<0.001	-
Number of agglomeration events to which equines have been sent			
Continuous	0.06	0.009	1.06 (1.06-1.10)
Shared food containers		0.01	3.74 (1.61-8.70)
Yes	1.32		
No	-		
Has strangles been diagnosed at the farm		0.01	3.20 (1.43-7.13)
Yes	1.16		
No	-		

Roc curve=71%, Hosmer test= p=0.85

Table 5. Multivariable logistic regression analysis of variables from the animal model associated with isolation of *S. equi*

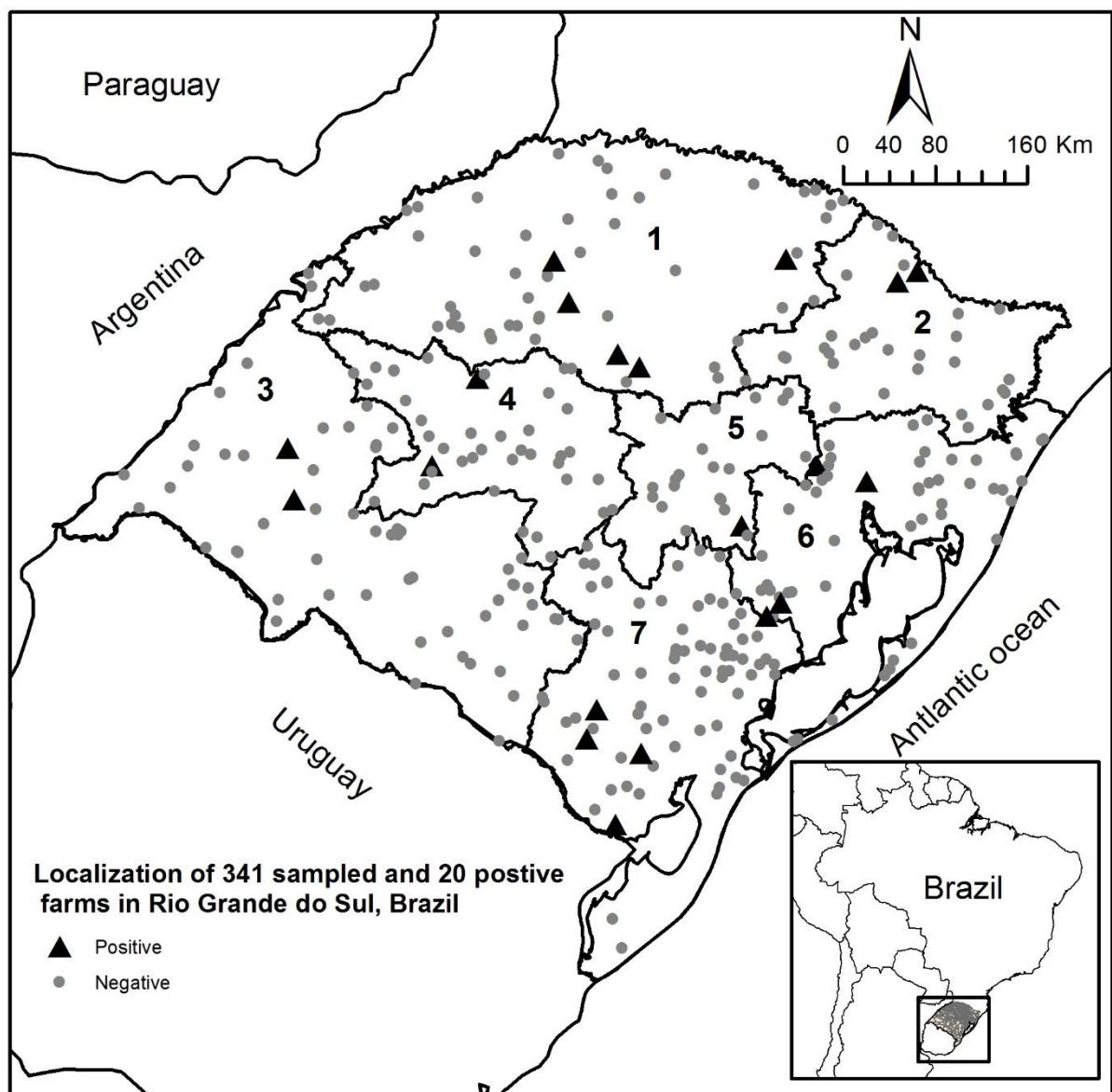| Variables	Estimate (β)	*P* – value	PR (CI: 95%)
Intercept	1.78	<0.001	-
Equine race			
Crioulo	-	-	-
Mangalarga marchador	-14.53	0.99	4.85(0 - +∞)
Thoroughbred	-14.53	0.99	4.85(0 - +∞)
Quarter Horse	2.02	<0.001	7.58(2.78-20.64)
Mixed breed	0.25	0.59	1.29(0.58-2.83)
Others	-14.53	0.99	4.85(0 - +∞)

Roc curve=73%, Hosmer test= p=0.74

Fig. 1. Location of sampled farms in Rio Grande do Sul state (Brazil) that were examined for *S. equi.* The names of the regions are indicated (1: Northwest Region, 2: Northeast Region, 3: Southwest Region, 4: West Central Region, 5: East Central Region, 6: Metropolitan Region, and 7: Southeast Region).

4 - CAPÍTULO 3

(Artigo submetido a revista Veterinary Microbiology)

seM alleles of *Streptococcus equi* subsp. *equi* in carrier horses

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ABSTRACT

Streptococcus equi subsp. *equi* (*S. equi*) is a beta hemolytic bacterium that causes equine adenitis, the main disease of the equine upper respiratory tract. M protein stands out among the main factors of virulence of *S. equi*, being responsible for its fixation in cells of the respiratory epithelium of the host, and the evasion of the immune system response. This protein presents mutations at the end of the N-terminal region of the sequence of the encoder gene (*seM*) which are used to differentiate the isolates from the characterization of *seM* alleles. Thus, this study aimed to identify *seM* alleles in equine carriers. For this purpose, 1010 samples of nasal swab collected from apparently healthy equines were cultivated in blood agar, from which twenty-four were identified positive by isolation and characterization of the morphotype. These samples were submitted to PCR and sequencing for characterization of alleles, being the same allele (*seM*-61) identified in all samples. These results support the hypothesis of the natural selection of alleles apparently more adapted to survive, persist and perpetuate within the studied population.

Keywords: Adenitis, strangles, M protein, *S.equi*

1. Introduction

Equine adenitis, popularly known as strangles, is the main disease of the upper respiratory tract of horses (Slater 2007). This highly contagious disease is characterized by purulent nasal discharge and regional lymphadenitis caused by *Streptococcus equi* subspecies *equi* (*S. equi*). Infection occurs after inhalation or ingestion of the agent that fixates, penetrates the mucosa, and then migrates to regional lymph nodes causing their fullness with consequent neck tourniquet (Timoney and Kumar, 2008).

S. equi is a beta hemolytic bacterium, Gram-positive Lancefield group C coccus that is highly adapted to the host (Boschwitz and Timoney, 1994). Approximately 10-20% of the horses that get sick and heal become persistent *S. equi* carriers, harboring the infectious agent in the respiratory tract, particularly in the guttural pouch, acting as a source of infection for healthy horses (Chanter *et al*., 2000; Newton *et al*, 1997). Carriers are asymptomatic and, from an epidemiological point of view, they act as a source of infection for other horses, thus continuing the propagation of the disease (Chanter *et al*., 2000; Newton *et al*., 1997; Newton *et al*., 2000).

M protein (seM) stands out among the major virulence factors of *S. equi*, , and it has antiphagocytic action due to fibrinogen and IgG binding, hindering bacterial opsonization, and preventing the deposition of the C3b complement component on the bacterial surface (Boschwitz and Timoney, 1994; Meehan *et al*, 2001). Molecular studies have demonstrated mutations in the sequence of the *seM* gene of *S. equi,* located in the 5' region (Chant *et al*., 2000; Anzai *et al*., 2005). This gene region has been used in to differentiate isolates by alleles characterization (Anzai *et al*., 2005) and to identify sources of outbreaks (Kelly *et al*., 2006; lvens *et al*, 2011; Parkinson *et al* 2011). Furthermore, variations in allele frequencies among

strains have become apparent over time, suggesting selection pressure in these strains (Kelly et al., 2006; lvens *et al*, 2011.).

In addition, a study conducted by Kelly *et al*. (2006), in England, demonstrated allele variation in samples collected from horses during the occurrence of the outbreak (*seM*-6) and samples collected from the same animals three months after recovery (bearing of *seM*-7), leading to the possibility of correlation between allelic variation and the determination of the carrier status.

Given the importance of a better understanding of equine adenitis and its etiological agent combined with the lack of studies on the frequency of *seM* alleles in apparently healthy horses, this study aimed to analyze and differentiate isolates of S. equi obtained from healthy horses in Rio Grande do Sul, Brazil, through the characterization of alleles based on the sequencing of the 5' region of the *seM* gene.

2. Materials and Methods

2.1 Isolation and identification of *S. equi*

Nasal swab samples (1010) were collected from healthy horses and submitted to the Bacteriology Laboratory of Universidade Federal de Santa Maria, in Santa Maria, Rio Grande do Sul-Brazil. The samples were cultured on blood agar (5% sheep blood), and incubated aerobically at 37°C for a period of 48 hours. Colonies morphologically compatible with *S. equi* were subcultured on blood agar plates for subsequent characterization by morphotype analysis and tests with sugar fermentation according to Macfaddin (2000) and Kuwamoto *et al*. (2001).

2.2 DNA extraction, PCR and sequencing

Bacterial cells identified as *S.equi* were suspended in 1 ml of Milli-Q water to conduct the DNA extraction through the CTAB (cetyltrimethyl ammonium bromide) protocol, preceded by a 5μL digestion of proteinase K (20 mg/ ml) at 37°C for 60 minutes according to Sambrook and Russell (2001). The nucleic acid was subsequently extracted with buffered phenol-chloroform. The precipitation was performed with isopropanol (overnight) and ethanol 70°GL. After drying, the pellet was resuspended in Milli-Q water.

Polymerase chain reaction (PCR) and primers ASW73 sense (5'-CAG AAAACTCGGAAGTGCTG-3') and ASW74 antisense (5'-ATT CGGTAAGAGCTTGACGC-3') were performed according to Kelly *et al*. (2006) for the amplification of the N-terminal region of the *seM* gene. The PCR reaction was performed in a final volume of 25μL, containing 5μL of GoTaq® Buffer, 10μM of each primer, 200μM of each deoxinucleotodyl triphosphate (dNTP), 1U of GoTaq® DNA Polymerase, 1μL template DNA(~50ng) and ultrapure water. The amplification was performed using an initial denaturation for 5 minutes at 95°C followed by 35 cycles of 30s at 95°C, 30s at 55°C, 40s at 72°C and a final 5-minute extension at 72°C. The amplification products were verified in 1% agarose gel stained with ethidium bromide (0.5μg mL-1).

The sequencing of the PCR products of all samples was performed in triplicate by ACTGene Análises Moleculares LTDA (Centro de Biotecnologia, UFRGS, Porto Alegre, RS) using the automated sequencer ABI PRISM 3100 Genetic Analyzer armed with 50 cm capillaries and POP6 polymer (Applied Biosystems).

2.3 Analysis of electropherograms obtained and characterization of alleles

Chromatograms obtained after sequencing were aligned and analyzed using Gap 4 software of the Staden package (Staden, 1996). The consensus nucleotide sequences obtained were compared with the sequence of M protein of strains available in GenBank (NCBI) to confirm genus species and subspecies (*S.equi*). Allele identification was performed with the MEGA 5 software and the alleles of M protein found were compared with the database PubMLST-SeM

(http://pubmlst.org/perl/bigsdb/bigsdb.pl?db=pubmlst_szooepidemicus_seqdef).

3. Results and discussion

Among the 1010 samples, 24 isolates were characterized as *S.equi*, all belonging to the *seM*-61 allele when compared to the sequences available to the PubMLST-SeM database, featuring these horses as carriers of the same allele. It is important to note that the identification of the *seM*-61 allele has been described in the UK by Ivens *et al*. (2011) and in Brazil by Libardoni *et al*. (2013), being the latter described in isolated horses with clinical disease.

The source or stimulus for allele *seM* variation is not yet completely elucidated, but it is known that, , virulence genes tend to present more mutations through immunological pressure as a form of resistance and evasion of the immune system, because such changes in the nucleotide sequence may lead or not to the modification of the protein component amino acid, of its three-dimensional organization, as well as the consequent alteration of epitopes (Kelly *et al*., 2006). In addition, many studies have shown positive immune selective pressure (at the protein level) by calculating the ratio of synonymous and nonsynonymous substitutions

of amino acids (dN / dS), especially in long-term infections, featuring the carrier status (Ivens *et al*, 2011; Kelly *et al*., 2006; Libardoni *et al*, 2013).

The identification of only one allele (*seM*-61) in 24 carrier horses leads to the hypothesis of natural selection of alleles that can more effectively evade from the immune system and for this reason settle and be identified with greater frequency within the equine population. In Brazil, Libardoni *et a*l., (2013) found the highest frequency of *seM*-61 allele in samples from Thoroughbred Racing horses, and *seM*-115 allele in Crioulo horses between the years of 1994 and 2010, showing that these alleles tend to settle in the equine population .This was also demonstrated by Ivens *et al*., (2011) between the years 2007-2010 in the United Kingdom, however, in view of the different population studied, a higher frequency of the alleles *seM*-6, *seM*-7 and *seM*-9 was found.

In this study, the medical history of the animals was not possible to be obtained prior to the collection in order to determine which *seM* allele of *S.equi* caused the outbreak and whether it was the same allele currently identified (*seM*-61). However, during the investigation of an outbreak in the UK, Kelly *et a*l. (2006) identified the same allele (*seM*-6) causing an outbreak of strangles in 11 horses, yet three months after the end of the outbreak three horses were identified as *S.equi* carriers with the allele differing from the one causing the outbreak (*seM*-7). Furthermore, Libardoni et al., (2013) showed allele variation in different outbreaks in the same property (B, M, O and Q), where the first outbreak occurred with *seM*-116, *seM*-61, *seM*-119 and *seM*-125 (with differences of 9 amino acids in relation to *seM*-61) respectively. However, *seM*-61 allele was identified in the second outbreak in all the properties. This suggests that either the gene may have been stimulated to mutate due to immune pressure, or there was a random mutation that may have been selected for giving more resistance to *S.equi*, enabling the carrier status. In addition, Parkinson *et al*., (2011) showed that alleles more frequently identified in horses in the UK are also prevalent in

countries of the European mainland, Spain, for instance, and, according to Anzai *et al*. (2005), strains with other alleles are predominant in the USA and Japan, as well as Brazil, as shown by Libardoni *et al*. (2013). It implies that more adapted alleles tend to survive, persist and perpetuate in larger amounts within the population.

A complete understanding of the meaning of *seM* variation of *S.equi* regarding virulence is not yet fully established. Timoney *et al*. (2010) showed that the N-terminal variation of M protein did not affect the binding of fibrinogen or the antibody-mediated opsonization, though it had great importance for mucosal immune responses via IgA and T-cells. In addition, a large proportion of antibody responses in convalescent horses is directed to the N-terminal of M protein (Timoney *et al*., 1998).

Another evasion mechanism demonstrated by Chanter *et al*. (2000) was the occurrence of truncation of M protein in *S.equi* in the guttural pouches of equine carriers, and these isolates show increased susceptibility to in vitro phagocytosis and less pathogenic potential, although they can cause the disease in horses when administered at a high bacterial dose. Two types of truncation of M protein have been identified so far. Chanter *et al*. (2000) showed deletions between nucleotides 363 and 441 of the M protein gene, and Parkinson *et al*. (2011) identified a nucleotide insertion changing the reading frame and resulting in a *stop codon* at position 134 of the gene. However, these events have not been observed in a previous study by Libardoni *et al*. (2013) and in this study.

Although most studies are directed to the *seM* gene as a marker to differentiate isolates, a number of other factors regarding the determination of the carrier status should be considered. Colonization factors such as adhesins, proteins binding to other compounds, pili and proteases should also be taken into account (Nobbs *et al*., 2014).

,Although our study involves the analysis of samples in only one diagnostics laboratory, the origin of the samples is representative of the entire state of Rio Grande do Sul

(Map 1), different from what was reported by Libardoni *et al.* (2013) in which clinical isolates from 21 properties were used. Another difference is that Libardoni *et al.*, (2013) found a wide variety of alleles while working with clinical samples, and in the present study the same allele was found in all horses, highlighting the difference in the sampling design, since this study is based on healthy horses, whereas the previous was based on clinical samples of nasal swab and content of abscesses.The data presented here provide information regarding the occurrence of single allele in different populations of apparently healthy horses, and represent material for future studies which involve populations at different stages of infection and colonization of *S.equi,* which not only include the influence of these determinants in the development of the carrier status, but also highlight the importance of characterizing seM alleles as molecular markers.

4. Conclusion

The presence of only one allele of the M protein gene (seM-61) was observed in the horse population infected with *S.equi*, showing no variation in alleles of carriers, what suggests that the most adapted alleles tend to remain within the population.

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5 - CONSIDERAÇÕES FINAIS

São necessários mais estudos que busquem identificar nos equinos que adoecem, quais alelos são isolados após a cura desses animais, e que identifiquem qual ou quais fatores levam as variações de alelos seM.

Associado a estudos de caracterização dos alelos seM, também devem ser realizados estudos que considerem outros fatores de *S. equi* relacionados a colonização, evasão do sistema imune e manutenção no hospedeiro.

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